Chain Length Distribution of Amylopectins of Double- and Triple-Mutants Containing the Waxy Gene in the Inbred Oh43 Maize Background* **

H. Fuwa, Osaka (Japan), D. V. Glover, West Lafayette (U. S. A.), K. Miyaura, N. Inouchi and Y. Konishi, Osaka (Japan), and Y. Sugimoto, Nishinomiya (Japan)

Starch granules were isolated from mature kernels of double- and triple-mutant combinations of the waxy (*wx*) gene with other starch-modifying genes in the inbred Oh43 maize background. The amylose content and the distribution of unit chain-length of amylopectin were determined by enzymatic-chromatographic methods. Starches of the mutants containing the *wx* gene comprised 100% amylopectin. Amylopectin of the amylose-extender;waxy (*ae wx*) mutant had an increased proportion of long B chains and decreased proportion of short B chains, compared with *wx* amylopectin, whereas amylopectin of the dull;waxy (*du wx*) mutant had a decreased proportion of long B chains and an increased proportion of short B chains. Therefore, the *ae wx* and *du wx* mutants amylopectins were novel. The A:B chain ratios for amylopectins examined, namely for *ae wx*, amylose-extender;waxy;floury-2 (*ae wx* fl_2), amylose-extender;waxy;sugary-1 (*ae wx* su_1), amylose-extender;waxy;sugary-2 (*ae wx* su_2), brittle-1;waxy (bt_1wx), dull;waxy (*du wx*), and sugary-2;waxy (su_2wx) amylopectins, were in a range of 1.1 to 1.4 and similar to the *wx* amylopectin. Thus, starches of double-mutant combinations of the *wx* gene with other starch-modifying genes are good sources for elucidating the fine structure of amylopectin, in regards to long- and short-B chains, which is affected by a single recessive gene coupled with the *wx* gene.

Kettenlängenverteilung der Amylopektine von Doppel- und Dreifach-Mutanten, die das wachsige (*wx*) Gen in der Inzucht-Maislinie Oh43 enthalten. Aus den reifen Körnern von doppelt- und dreifachmutanten Kombinationen des wachsigen (*wx*) Gens mit anderen stärkemodifizierenden Genen der Inzuchtlinie Oh43 von Mais wurden die Stärkekörner isoliert. Der Amylosegehalt und die Einheits-Kettenlängenverteilung wurden durch enzymatische Methoden bestimmt. Die Stärken der von *wx*-Gen enthaltenden Mutanten bestanden zu 100% aus Amylopektin. Das Amylopektin der Amyloseextender-Wachs-Mutante (*ae wx*) hatte im Vergleich zu *wx*-Amylopektin einen erhöhten Anteil an langen und einen verringerten Anteil an kurzen B-Ketten, demgegenüber das Amylopektin der stumpfwachsigen (*du wx*) Mutante einen verringerten Anteil an langen und einen erhöhten Anteil an kurzen B-Ketten aufwies. Somit waren die Amylopektine der *ae wx* und der *du wx*-Mutanten neuartig. Das Verhältnis von A- zu B-Ketten der untersuchten Amylopektine, nämlich *ae wx*, Amyloseextender-Wachs-Mehl-2 (*ae wx* fl_2), Amyloseextender-Wachs-Zucker-1 (*ae wx* su_1), Amyloseextender-Wachs-Zucker-2 (*ae wx* su_2), Spröde-1-Wachs (bt_1 *wx*), Stumpf-Wachs (*du wx*) und Zucker-2-Wachs (su_2 *wx*) lag im Bereich von 1,1 bis 1,4 und war ähnlich dem des *wx*-Amylopektins. Daher sind Stärken der doppelt-mutanten Kombinationen des *wx*-Gens mit anderen stärkemodifizierenden Genen gut zur Aufklärung der Feinstruktur des Amylopektins hinsichtlich des Verhältnisses langer zu kurzen B-Ketten geeignet, das durch ein einzelnes Gen, gekoppelt mit dem *wx*-Gen, beeinflußt wird.

1 Introduction

Several endosperm genes, which modify structure and properties of endosperm starch, have been identified in maize (*Zea mays* L.) [1]. Amylose-extender (*ae*) gene is associated with high amylose, high intermediate fraction and a novel amylopectin [2, 3]. Dull (*du*), sugary-1 (su_1), sugary-2 (su_2) and brittle-1 (bt_1) genes in the inbred Oh43 background are also associated with high amylose. The waxy (*wx*) gene produces 100% amylopectin, which has a similar chain-length distribution to amylopectin of the normal (nonmutant) counterpart as demonstrated by two enzymatic-chromatographic methods [4]. Moreover, the *wx* mutant of maize is epistatic to all other known mutants relative to the lack of accumulation of amylose [1–5]. Therefore, starches of double- and triple-mutant combinations of the *wx* gene with other starch-modifying genes, for example starches of the *ae wx*, bt_1wx, *du wx*, su_1wx mutant, comprise solely amylopectin, and very little amylose.

The present paper describes the chain-length distribution of various amylopectins and properties of starches of double- and triple-mutant combinations of the *wx* gene with either *ae*, bt_1, *du*, su_1, or su_2 genes in the inbred Oh43 by the two enzymatic-chromatographic methods.

2 Materials and Methods

2.1 Maize seeds

The following eight double- or triple-endosperm mutants in the inbred Oh43 maize (*Zea mays* L.) background were used; *ae wx*, *ae wx* fl_2, *ae* su_1wx, *ae* su_2wx, bt_1wx, *du wx*, su_1wx, and su_2wx. The materials were grown at the Purdue University Agronomy farm.

2.2 Enzymes

Crystalline *Pseudomonas* isoamylase (EC 3.2.1.68) and *Aerobacter* pullulanase (EC 3.2.1.41) were purchased from

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Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Pullulanase was purified by cyclohexaamylose-coupled Sepharose 6B affinity gel chromatography [6] and freed of α-amylase. β-Amylase (EC 3.2.1.2), a product of Sigma Chemical Co., St. Louis, MO, U. S. A., was purified by the method of Marshall and Whelan [7] and by affinity gel chromatography [6], and freed of α-amylase and α-glucosidase.

2.3 Preparation of starch granules

Starch granules were prepared from maize endosperms by *Schoch*'s method [8] and purified by *Watson*'s method [9].

2.4 Measurement of iodine absorption spectra

Absorption curves of starch-iodine complexes were recorded with a Hitachi 220-type recording spectrophotometer with mixtures containing 1 mg of starch, 2 mg of iodine and 20 mg of potassium iodide per 25 ml [10].

2.5 Debranching of starch with isoamylase and fractionation of debranched materials

Maize starches were debranched with crystalline *Pseudomonas* isoamylase by the method of *Ikawa* et al. [2]. Debranched materials were fractionated by gel filtration on either Sephadex G-75 column or columns of Toyopearl HW55SF and HW50SF (Toyosoda, Co. Ltd., Tokyo, Japan) connected in series [2, 3]. The range of fractions I, II and III, and an intermediate fraction was divided according to the wavelengths of absorption maxima (λ_{max}) of absorption spectra of iodine-glucan complexes in each tube. Fr. I, $\lambda_{max} \geqq 620$ nm, intermediate Fr., 620 nm $> \lambda_{max} \geqq 600$ nm, Fr. II 600 nm $> \lambda_{max} \geqq 540$ nm, and Fr. III, 540 nm $> \lambda_{max}$.

2.6 Preparation of amylopectin β-limit dextrin.

Starch was digested with crystalline β-amylase and amylopectin β-limit dextrin was purified by the method of *Inouchi* [4, 11].

2.7 Debranching of amylopectin β-limit dextrin and fractionation of debranched β-limit dextrin

Amylopectin β-limit dextrin was debranched successively with isoamylase and pullulanase, and debranched materials were fractionated by gel filtration on a column of Toyopearl HW50SF by the method of *Inouchi* [4, 11]. Fraction I_β (Fr. I_β) was divided according to the wavelength of λ_{max} ($\geqq 600$ nm) of absorption spectra of iodine-glucan complexes. Fractions II_β, III_β, and IV_β were divided according to the range of average chain lengths ($\overline{CL}$). Namely, Fr. II_β, $\overline{CL} \geqq 20$, Fr. III_β, $20 > \overline{CL} \geqq 4$, and Fr. IV_β, $4 > \overline{CL} \geqq 2$.

2.8 Analytical methods

The carbohydrate content was determined by the phenol-sulfuric acid method [12]. Reducing end groups were determined by the *Park-Johnson* method [13] as modified by *Hizukuri* et al. [14]. The average chain lengths ($\overline{CL}$) were calculated from the amount of carbohydrate and the number of reducing ends.

2.9 Other methods

The methods for determination of starch granule susceptibility to amylase were reported previously [15]. X-ray diffractometry was performed by the method of *Hizukuri* et al. [16] and the procedure for differential scanning calorimetry (DSC) was described elsewhere [17].

3 Results

3.1 Structure and properties of amylopectins of maize double-mutant combinations of the waxy gene with either brittle-1, dull, sugary-1 or the sugary-2 gene

Some characteristics of absorption curves of starch-iodine complexes shown in the second and third columns of Table 1 strongly suggest that endosperm starches of these maize double-mutant combinations were waxy types. Fraction I, which was regarded as amylose of nonwaxy starch, was absent in the starches (Table 1), that is, their composition was all amylopectins. The ratio of fraction III to intermediate fraction plus fraction II is the ratio of short B chains plus A chains to long B chains of amylopectin and represents one of the structural characteristics of amylopectin (Table 1). The ratios for *bt*$_1$*wx*, *su*$_1$*wx*, and *su*$_2$*wx* starches were slightly higher than that for the *wx* starch. The *du wx* starch had the highest ratio among starches shown in Table 1.

In order to characterize the detailed distribution of unit-chain length of the amylopectins, β-amylase limit dextrins were prepared from the *bt*$_1$*wx*, *du wx*, and *su*$_2$*wx* starches, the β-limit dextrins were debranched successively with isoamylase and pullulanase, and the debranched-materials were fractionated by gel filtration. Table 2 shows number on a percentage basis (percentage of reducing ends) of the debranched materials. Among each fraction (Fr.), Fr. I_β corresponded to the β-limit dextrin of amylose. Frs. II_β and III_β corresponded to the internal chains of the long B and short B chains of amylopectin, respectively, Fr. IV_β corresponded to the internal chains of the A chains, and the number percentage of the fraction represented the molar ratio of the A chains in the original

Table 1.
Some Properties of Endosperm Starches and Their Isoamylase-Debranched Materials of Single- and Double-Mutants of Inbred Oh43 Maize with the Waxy Gene.

	λ_{max} (nm)	Blue value (at 680 nm)	Distribution of starch materials (%)*				Fr. III / Fr. II	Fr. III / Int. Fr. + Fr. II
			Fr. I	Intermed. Fr.	Fr. II	Fr. III		
wx	527	0.070	–	4.9	24.5	70.6	2.9	2.4
bt$_1$ *wx*	525	0.060	–	2.2	22.7	75.2	3.3	3.0
du wx	520	0.066	–	1.0	18.0	81.0	4.5	4.3
su$_1$ *wx*	520	0.054	–	2.7	20.7	75.2	3.7	3.2
su$_2$ *wx*	530	0.055	–	2.8	19.7	77.5	3.5	3.4

* Each fraction (Fr.) was divided according to the following range of λ_{max} values of iodine-glucan complexes: Fr. I $\lambda_{max} \geqq 620$ nm, Intermediate Fr. 620 nm $> \lambda_{max} \geqq 600$ nm, Fr. II 600 nm $> \lambda_{max} \geqq 540$ nm, and Fr. III 540 nm $> \lambda_{max}$.

Table 2.
Percentages of Reducing Ends of Materials of β-Limit Dextrins Debranched with Isoamylase and Pullulanase and Some Characteristics of β-Limit Dextrins Prepared from Endosperm Starches of Single- and Double-Mutants of Inbred Oh43 Maize with the Waxy Gene*.

	Fr. I_β (%)	Fr. II_β (%)	Fr. III_β (%)	Fr. IV_β (%)	Fr. III_β / Fr. II_β	A / B
wx	0.0	10.3	33.2	56.5	3.2	1.3
bt$_1$ *wx*	0.0	11.1	34.2	54.7	3.1	1.2
du wx	0.0	6.6	40.3	53.1	6.1	1.1
su$_2$ *wx*	0.0	9.9	32.5	57.6	3.3	1.3

* Fraction I_β (Fr. I_β) was divided according to the wavelength of λ_{max} ($\geqq$ 600 nm) of absorption spectra of glucan-iodine complexes in each tube. Fractions II_β, III_β and IV_β were divided according to the average chain lengths ($\overline{CL}$). Namely, Fr. II_β $\overline{CL} \geqq 20$, Fr. III_β $20 > \overline{CL} \geqq 4$, and Fr. IV_β $4 > \overline{CL} \geqq 2$.

amylopectin [4, 11]. Starches of the double-mutant combinations of *wx* with either *bt*$_1$, *du*, or *su*$_2$ had a similar A:B ratio (1.1 to 1.3) to that for the *wx* starch (1.3). The ratio of short B to long B chains of the *du wx* amylopectin (Fr. III_β:Fr. II_β ratio, 6.1) was highest among the starches examined. Thus *du wx* mutant has a novel amylopectin compared to the *wx* mutant.

Figure 1 shows X-ray diffractograms of the *du wx*, *su*$_2$*wx*, and *bt*$_1$*wx* starches. The diffractograms were of the type-A pattern, which is typical of cereal starches, although that of the *su*$_2$*wx* starch had weak and broad diffraction peaks similar to those of the *su*$_2$ starch [2, 18].

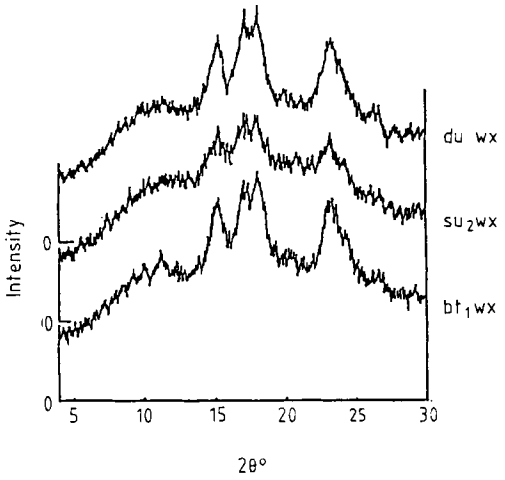

Fig. 1. X-ray diffractograms of starches of the double-mutant combinations of waxy (*wx*) gene with either dull (*du*), sugary-2 (*su*$_2$), or brittle-1 (*bt*$_1$) gene in inbred Oh43 maize background.

The DSC thermograms of starches of the double-mutant combinations with the *wx* gene shifted to the higher temperature ranges compared with those of starches of the single-mutants which were involved in the respective gene combination with the *wx* gene. T_c values of the double-mutant starches were similar to or higher than that of the *wx* starch (Table 3). The ΔH value of the *su*$_2$*wx* starch was the smallest compared with those of the other double- and single-mutant starches, which might suggest that the amount of hydrogen bonding of the *su*$_2$*wx* starch was less than those of the other starches.
Starches of the nonmutant, and *bt*$_1$*wx*, *du wx*, *su*$_1$*wx*, and *su*$_2$*wx* mutant were, in that order, increasingly more susceptible to the action of crude glucoamylase of *Rh. amagasakiens* (data were not shown) as reported previously [19] except for the *du wx* starch.

Table 3.
The Endotherm Characteristics and Heats of Gelatinization of Endosperm Starches of Several Mutants of Inbred Oh43 Maize*.

	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (cal/g)
bt$_1$ *wx*	67	71	81	3.5
du wx	65	72	83	2.9
su$_1$ *wx*	63	68	76	2.5
su$_2$ *wx*	62	67	81	0.5
wx	67	75	80	3.7
bt$_1$	61	66	73	1.2
du	67	71	76	2.9
su$_1$	62	66	69	1.8
su$_2$	54	58	64	1.2

* T_o, T_p and T_c, and ΔH are onset, peak and conclusion temperatures for gelatinization, and heat of gelatinization, respectively.

3.2 Structure and properties of maize amylopectins of double- and triple-mutant combinations of waxy gene with amylose-extender and some other genes.

In a previous paper [2], we showed the *ae wx* starch of the Oh43 inbred consists of amylopectin having a loosely-branched structure and a longer average chain-length than that of the *wx* starch as shown by the enzymatic-chromatographic methods. To elucidate the detailed distribution of unit-chain length of the *ae wx* amylopectin, β-amylase limit dextrins were prepared from the *ae wx*, *ae wx fl*$_2$, *ae wx su*$_1$, and *ae wx su*$_2$ starches and debranched with isoamylase and pullulanase, and the debranched-materials were fractionated by gel filtration. Table 4 shows that amylopectins of the *ae wx* series have similar proportions of the A chains (Fr. IV_β) to the *wx* amylopectin, however, the amylopectins of the double- and triple-mutants have increased proportions of the long B chains (Fr. II_β), namely about twice as much as that of the *wx* amylopectin, and have decreased proportions of the short B chains (Fr. III_β), namely about three-fourths that of the *wx* amylopectin.
Therefore, amylopectins of the double- and triple-mutants showed A:B ratios of 1.3 to 1.4 which are similar to the ratio for the *wx* amylopectin (1.3). However, the Fr. III_β:Fr. II_β ratios for the double- and triple-mutants were 1.1 to 1.4, whereas that for the *wx* amylopectin was 3.2.
Starches of the double- and triple-mutants of *ae wx* showed X-ray diffractograms of the type-B pattern, which is typical of potato starch, although they had weak and broad diffraction peaks compared with those of potato starch (data are not shown). The gelatinization temperatures and ΔH values for

Table 4.
Percentages of Reducing Ends of Materials of β-Limit Dextrins Debranched by Isoamylase and Pullulanase and A:B Chain Ratio of Starches of Double- and Triple-Mutants of Inbred Oh43 Maize with Amylose-Extender (*ae*) and Waxy (*wx*) Genes*.

	Fr. I_β (%)	Fr. II_β (%)	Fr. III_β (%)	Fr. IV_β (%)	Fr. III_β / Fr. II_β	A / B
wx	0.0	10.3	33.2	56.5	3.2	1.3
ae wx	0.0	20.8	23.5	55.7	1.1	1.3
ae wx fl$_2$	0.0	19.9	23.2	56.9	1.2	1.3
ae wx su$_1$	0.0	18.5	25.0	56.5	1.4	1.3
ae wx su$_2$	0.0	18.4	22.7	58.9	1.2	1.4

* See the legend to Table 2.

starches of the *ae wx* mutants were not determined by DSC, since the low and broad peak extended into the region beyond 100°C.

4 Discussion

In previous papers [2, 3], we showed by way of two enzymatic-chromatographic methods [4, 11], that *wx* produces 100% amylopectin and the *wx* amylopectin has a similar distribution of unit chain-length to amylopectin of the normal (nonmutant) counterpart. The *wx* mutant of maize is known to be epistatic to all other known mutants relative to the lack of accumulation of amylose [1–5]. Therefore, starches of double-mutant combinations of the *wx* with other starch-modifying genes, for example the *ae wx*, bt_1wx, *du wx*, and su_2wx starch, consist solely of amylopectin and are good sources for elucidating the fine structure of an amylopectin which is affected by a single recessive gene coupled with the *wx* gene.

Applying the enzymatic-chromatographic methods to amylopectins of the double-mutant combinations of the *wx* gene with either *ae*, bt_1, *du*, or su_2 gene in the inbred Oh43 background, two novel amylopectins of the *ae wx* and *du wx* mutants were differentiated from the *wx* amylopectin by distributions of the unit chain-length (Tables 1, 2, and 4). The *ae wx* amylopectin had an increased proportion of the long B chains (102% more) and a decreased proportion of the short B chains (39% less) as compared with the *wx* amylopectin, whereas the *du wx* amylopectin had a decreased proportion of the long B chains (36% less) and an increased proportion of the short B chains (21% more). The bt_1wx and su_2wx amylopectins had similar proportions of each of the long and short B and A chains to that of the *wx* amylopectin.

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Addresses of authors: Professor *Hidetsugu Fuwa*, Ms. *K. Miyaura*, Dr. *Naoyoshi Inouchi*, Dr. *Y. Konishi*, Department of Food and Nutrition, Osaka City University, 138, 3–3, Sugimoto, Sumiyoshi, Osaka 558 (Japan).

Professor *David. V. Glover*, Department of Agronomy, Purdue University, West Lafayette, IN 47907 (U.S.A.).

Dr. *Y. Sugimoto*, Department of Home Science, Mukogawa Women's University, Ikebiraki, Nishinomiya 663 (Japan).

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Varietal Differences in Physicochemical Properties of Waxy Rice Starch

B. O. Juliano and R. M. Villareal, Los Baños, Laguna (Philippines)

High-gelatinization temperature (GT) waxy rice starch amylopectin has higher sedimentation coefficient than low-GT waxy rice amylopectin. Gel filtration on Sepharose CL-2B and TSK-Gel G-6000PW also showed higher mean molecular weights for high-GT amylopectins than for low-GT amylopectins. The harder texture of cooked rice products from high-GT waxy rices, compared to the texture of products from low-GT waxy rices, may be due to the higher molecular weight of their amylopectins.

Varietätsbedingte Unterschiede bei den physikalisch-chemischen Eigenschaften von wachsiger Reisstärke. Amylopektin aus wachsiger Reisstärke von hoher Verkleisterungstemperatur (High-GT) besitzt einen höheren Sedimentations-Koeffizienten als das von niedrig verkleisternder (Low-GT) wachsiger Reisstärke. Gelfiltration auf Sepharose CL-2B und TSK-Gel G 6000 PW zeigte auch höhere mittlere Molekulargewichte für High-GT-Amylopektine als für Low-GT-Amylopektine. Die härtere Struktur von gekochten Reisprodukten aus High-GT-Wachsreis im Vergleich zur Textur der Produkte aus Low-GT-Wachsreis dürfte auf dem höheren Molekulargewicht ihrer Amylopektine beruhen.

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